MicroReview

Class A PBPs: it is time to rethink traditional paradigms

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ABSTRACT

Until recently class A penicillin-binding proteins (aPBPs) were the only enzymes known to catalyze glycan chain polymerization from lipid II in bacteria. Hence, the discovery of two novel lipid II polymerases, FtsW and RodA, raises new questions and has consequently received a lot of attention from the research community. FtsW and RodA are essential and highly conserved members of the divisome and elongasome, respectively, and work in conjunction with their cognate class B PBPs (bPBPs) to synthesize the division septum and insert new peptidoglycan into the lateral cell wall. The identification of FtsW and RodA as peptidoglycan glycosyltransferases has raised questions regarding the role of aPBPs in peptidoglycan synthesis and fundamentally changed our understanding of the process. Despite their dethronement, aPBPs are essential in most bacteria. So, what is their function? In this review we discuss recent progress in answering this question and present our own views on the topic.

1 | INTRODUCTION

A considerable number of proteins are involved in building and splitting the bacterial cell wall during cell growth and division (Table 1). The peptidoglycan part of the wall consists of a continuous network of circumferentially oriented glycan chains crosslinked by short peptide bridges. It is assembled from lipid-linked disaccharide-peptide subunits (Lipid II), which are flipped across the cytoplasmic membrane by MurJ (Ruiz, 2008; Sham et al., 2014; Meeske et al., 2015). Peptidoglycan glycosyltransferases polymerize the disaccharide units into glycan strands, while peptidoglycan transpeptidases cross-link and process the peptide side chains to produce a mesh-like structure (Vollmer et al., 2008a; Lovering et al., 2012). The disaccharide units consist of β-1,4-linked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). All bacterial peptidoglycan contains the same carbohydrate backbone of alternating MurNAc and GlcNAc residues, but the length of the glycan strands varies between species (Vollmer & Seligman, 2010). In addition, the glycan strands can be further modified by addition or removal of acetyl residues (Vollmer & Tomasz, 2000; Cristóstomo et al., 2006; Vollmer, 2008; Moynihan & Clarke, 2010). The pentapeptide linked to MurNAc is not universally conserved among bacteria. Its amino acid composition varies, especially at the second and third positions (Vollmer et al., 2008a). In *Streptococcus pneumoniae* and many other Gram-positive bacteria, the pentapeptide chain has the following sequence: L-Ala-D-iso-

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65 Gln-L-Lys-D-Ala-D-Ala. Instead of D-iso-Gln and L-Lys, *Escherichia coli* for instance has D-
66 iso-Glu and meso-diaminopimelic acid (meso-DAP) in the corresponding positions (Vollmer
67 et al., 2008a; Morlot et al., 2018). In most bacterial species, pentapeptides from different glycan
68 strands are cross-linked by transpeptidation between the fourth D-alanine residue of the donor
69 chain and the third residue (e. g. Lys or meso-DAP) of the acceptor chain. Alternatively, in
70 some species the stem peptides can be linked together by means of interpeptide bridges, which
71 in the case of *S. pneumoniae* consists of L-Ser-L-Ala or L-Ala-L-Ala (Vollmer et al., 2019).
72 The enzymes catalyzing the transpeptidation reaction are called penicillin-binding proteins
73 (PBPs). PBPs come in three different classes; A, B and C (Table 1) (Sauvage et al., 2008).
74 aPBPs are bifunctional enzymes that catalyze transglycosylation as well as transpeptidation,
75 while bPBPs are monofunctional and possess only transpeptidase activity. Class C PBPs
76 (cPBPs) are peptidoglycan hydrolases with D,D-carboxypeptidase or endopeptidase activity.
77 They primarily regulate the degree of crosslinking by removing the terminal D-Ala from
78 pentapeptide chains.

79 Until recently, the accepted paradigm was that bacteria produce only one type of
80 enzyme capable of synthesizing glycan strands from lipid II, namely aPBPs. Consequently, it
81 was believed that bacterial peptidoglycan synthesis is completely dependent on the
82 glycosyltransferase activity of these bifunctional PBPs, although one exception was noted
83 (McPherson & Popham, 2003). It therefore aroused considerable interest when it was reported
84 that FtsW and RodA, two essential proteins belonging to the SEDS family, also use lipid II as
85 substrate to polymerize new glycan strands (Meeske et al., 2016; Emami et al., 2017; Taguchi
86 et al., 2019). These proteins were originally reported to be lipid II flippases (Mohammadi et
87 al., 2011), a function later assigned to MurJ (Sham et al., 2014). However, it remains a
88 possibility that FtsW and RodA have a double role, i.e. have flippase as well as
89 glycosyltransferase activity (Egan et al., 2020). To synthesize peptidoglycan, FtsW and RodA
90 work in conjunction with monofunctional bPBPs (Meeske et al., 2016; Emami et al., 2017;
91 Taguchi et al., 2019; Sjodt et al., 2020). It is now firmly established that FtsW and RodA
92 together with their cognate transpeptidase partners form the core peptidoglycan synthesizing
93 machineries of the divisome and elongasome, respectively. The recent shift in our
94 understanding of peptidoglycan synthesis has important implications. It strongly suggests that
95 aPBPs are not the central players in septal cross-wall synthesis and cell elongation. What, then,
96 could be the role of aPBPs in the construction of the bacterial cell wall?

Over the past decades a vast amount of information on aPBPs from various bacterial species has been published. For the sake of simplicity and clarity, this short review will mainly focus on the aPBPs of *S. pneumoniae* and *E. coli* as representatives of Gram-positive and Gram-negative bacteria, respectively.

2 | THE VARIABLE ESSENTIALITY OF aPBPs

aPBPs seem to be present in most peptidoglycan-producing bacteria. Hence, they must perform an important function. However, their numbers vary between bacterial species (Table 1). *B. subtilis* produces four different aPBPs, *S. pneumoniae* and *E. coli* have three, while *S. aureus* survives and thrives with only one (Sauvage et al., 2008). Interestingly, bacteria producing several different aPBPs, often need only one of them for survival. In *S. pneumoniae*, single knockouts of *pbp1a*, *pbp2a* and *pbp1b* can be obtained, but it is not possible to construct a *pbp1a*/*pbp2a* double-knockout strain (Paik et al., 1999). The pneumococcus must therefore produce either PBP1a or PBP2a to be viable. PBP1b, on the other hand, cannot substitute for the other two and is not essential. Similarly, in *E. coli*, PBP1c is dispensable while the bacterium must produce either PBP1a or PBP1b to survive. Studies on the properties of aPBPs from model bacteria such as *S. pneumoniae*, *B. subtilis* and *E. coli* have revealed that many bacterial aPBPs are non-essential, while others have partly overlapping functions. Notably, it has been reported that *B. subtilis* as well as *Enterococcus faecalis* are viable even when all their aPBPs have been deleted (McPherson & Popham, 2003; Arbeloa et al., 2004). Deletion of the bifunctional PBPs in these species led to a significant increase in generation time, and to a modest decrease in cross-linking of their peptidoglycan. In the case of *B. subtilis*, the quadruple class A-less mutant was obtained at a near normal transformation frequency, indicating that the survival of this mutant does not depend on the acquisition of additional suppressor mutations (McPherson & Popham, 2003). Another interesting case is *Chlamydia trachomatis,* which produces peptidoglycan but not aPBPs. It does, however, harbor genes encoding bPBPs and SEDS proteins (Meeske et al., 2016; Cox et al., 2020). The same holds true for members of *Francisella*, *Wolbachia* and some other genera, demonstrating that bPBPs and SEDS proteins are more widely conserved than aPBPs (Meeske et al., 2016). Thus, although aPBPs play an important and usually indispensable role in the biosynthesis of bacterial peptidoglycan, SEDS proteins together with bPBPs seem to constitute the basic cell-wall-building machinery.

3 | PROPERTIES OF PNEUMOCOCCAL aPBPs

In addition to the three aPBPs (PBP1a, PBP1b and PBP2a), *S. pneumoniae* produces two bPBPs (PBP2x and PBP2b) and a single cPBP (PBP3) (Sauvage et al., 2008). PBP2x and PBP2b are essential transpeptidases (Kell et al., 1993) that work together in pairs with FtsW and RodA in the divisome and elongasome, respectively (Perez et al., 2019; Taguchi et al., 2019; Sjodt et al., 2020). PBP3 (DacA) functions as a D,D-carboxypeptidase that converts pentapeptide into tetrapeptide moieties by cleaving the terminal D-alanyl-D-alanine bond (Hakenbeck & Kohiyama, 1982; Severin et al., 1992). Presumably, PBP3 regulates the extent of crosslinking between glycan strands by limiting the amount of D-alanyl-D-alanine donor groups required for transpeptidation (Morlot et al., 2004; Barendt et al., 2011). The enzyme is distributed across the entire cell surface but is absent from the future division site (Morlot et al., 2004). Deletion of PBP3 gives rise to severe morphological defects including misplaced division septa (Schuster et al., 1990; Barendt et al., 2011).

PBP1a primarily localizes to the midcell of *S. pneumoniae* (Land et al., 2013). The divisome as well as the elongasome operate in this region. Several lines of evidence connect PBP1a with proteins involved in lateral cell wall elongation. The essentiality of MreC, MreD, RodZ and MltG is suppressed in a *S. pneumoniae* D39 strain lacking PBP1a (Land & Winkler, 2011; Fenton et al., 2016; Tsui et al., 2016). MreC, MreD, RodZ and MltG are all associated with the pneumococcal elongasome. MltG is a lytic transglycosylase, while MreC, MreD and RodZ are structural elements required to assemble a functional elongasome in rod-shaped and ovoid bacteria (Tsui et al., 2016; Stamsås et al., 2017; Winther et al., 2021). Similarly, a polytopic membrane protein named CozE (for coordinator of zonal elongation) can be deleted in a Δ*pbp1a* background, but not in a Δ*pbp2a* background (Fenton et al., 2016). PBP1a has been shown to directly interact with MreC, MreD, CozE and its paralog CozEb, all of which appear to be involved in controlling the activity of PBP1a (Land & Winkler, 2011; Fenton et al., 2016; Stamsås et al., 2020). As experimental data suggest that PBP1a forms a complex with the four above-mentioned proteins, PBP1a is assumed to be part of the pneumococcal elongasome. This is in accordance with the observation that PBP1a, PBP2b, and MreC colocalizes throughout the division cycle. In contrast, PBP2x colocalizes with PBP1a during the early stages of pneumococcal cell division, but at later division stages these PBPs occupy different positions in constricting division septa (Land et al., 2013; Tsui et al., 2014).

By screening for mutants synthetically lethal with a *pbp1a* deletion, Fenton and co-workers (2018) identified a protein termed MacP for membrane-anchored cofactor of PBP2a. MacP was shown to form a complex with PBP2a, and to be required for its *in vivo* function. Furthermore, MacP is phosphorylated by StkP, a eukaryotic-type serine-threonine kinase which is a regulator of cell division and morphogenesis. Pneumococcal StkP possesses a cytoplasmic catalytic domain and an extracellular PASTA domain consisting of four repeats. The PASTA domain is required for midcell localization of StkP and is involved in cell division as well as ligand sensing. It has been reported to sense muropeptides, lipid II levels and to control septal cell wall thickness (Maestro et al., 2011; Beilharz et al., 2012; Fleurie et al., 2012; Hardt et al., 2017; Zucchini et al., 2018; Fenton et al., 2018; Sun & Garner, 2020). MacP apparently constitutes a link between signals sensed by StkP and peptidoglycan synthase activity. Similar to deletion of the *macP* gene, deletion of *gpsB* is synthetically lethal with a *Δpbp1a* mutation, but not with *Δpbp2a* and *Δpbp1b* mutations. GpsB interacts with PBP2a, MreC and other cell wall and cell cycle proteins. It is an adaptor protein that regulates septal and peripheral peptidoglycan synthesis in *S. pneumoniae* and other low-GC Gram-positive bacteria (Fleurie et al., 2014; Rued et al., 2017; Cleverley et al., 2019). It is of interest to note that deletion of *pbp1a* reduce cell size in the D39 strain, while cell dimensions are not significantly changed in cells lacking *pbp2*a (Land & Winkler, 2011). This demonstrates that the two PBPs affect the morphology of the pneumococcal cell differently. Furthermore, considering that PBP1a and PBP2a are regulated by and interact with different proteins, the synthetic lethality of the *pbp*1a/*pbp*2a double mutation cannot be explained by simple functional redundancy. In sum, experimental data strongly indicate that PBP1a and PBP2a carry out overlapping functions as well as functions specific to each PBP.

aPBPs were recently shown to function autonomously *in vivo* in *S. pneumoniae* (Straume et al., 2020). It was discovered that bifunctional PBPs are still active in cells lacking a functional divisome or elongasome, demonstrating that they can operate independently of the two multiprotein complexes. This insight derives from experiments performed with a peptidoglycan hydrolase CbpD, which splits pneumococcal cells at the septum and in a poorly understood way is able to differentiate between peptidoglycan synthesized by the divisome (PBP2x/FtsW) and aPBPs. It was demonstrated that the activity of pneumococcal PBP1a or PBP2a is required to establish resistance against the lytic activity of CbpD. The finding that PBP2x/FtsW-synthesized peptidoglycan is sensitive to CbpD while class A-synthesized peptidoglycan is resistant, shows that the two types of peptidoglycan must differ in composition

193 and/or architecture. Furthermore, it was demonstrated that peptidoglycan synthesis by aPBPs
194 lags a few minutes behind the synthesis carried out by the PBP2x/FtsW machinery (Straume et
195 al., 2020). Together, these facts show that mature pneumococcal peptidoglycan is synthesized
196 by three independent entities; the divisome, the elongasome and the bifunctional aPBPs.
197 Lateral cell wall expansion and synthesis of the septum are carried out by the elongasome and
198 divisome, respectively, while the exact function of aPBPs is still a matter of debate.

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4 | PROPERTIES OF *E. COLI* aPBPs

201 The peptidoglycan wall of a Gram-negative bacterium, such as *E. coli,* differs fundamentally
202 from the peptidoglycan wall of Gram-positive bacteria (Egan et al., 2020). It is only one or a
203 couple of layers thick and is surrounded by an outer membrane composed of phospholipids and
204 lipopolysaccharide (Turner et al., 2013; Sperandeo et al., 2017). The genome of *E. coli* encodes
205 12 PBPs: three class A (PBP1a, PBP1b and PBP1c), two class B (PBP2 and PBP3) and seven
206 class C (PBP4, PBP4b, PBP5, PBP6, PBP6b, PBP7 and AmpH) PBPs (Table 1) (Sauvage et
207 al., 2008). The essential bPBPs, PBP2 and PBP3, are closely associated with the SEDS family
208 peptidoglycan polymerases of *E. coli*. The FstW/PBP3 pair makes up the core peptidoglycan
209 synthesizing machinery of the divisome, while RodA/PBP2 have a corresponding function in
210 the elongasome (Cho et al., 2016; Leclercq et al., 2017). Six of the cPBPs (PBP4, PBP4b,
211 PBP5, PBP6, PBP6b and AmpH) have D,D,-carboxypeptidase activity, while three (PBP4,
212 PBP7 and AmpH) have D,D-endopeptidases activity (Denome et al., 1999; Ghosh et al., 2008;
213 Typas et al., 2012; Pazos and Peters, 2019). It is not clear whether PBP4 and AmpH, which
214 have dual activities, carry out both activities *in vivo*, but enzymological data support that PBP4
215 primarily functions as a D,D-endopeptidases (Korat et al., 1991; Meberg et al., 2004; González-
216 Leiza et al., 2011). D,D-endopeptidases cleave the bond between D-Ala and meso-DAP in
217 cross-linked stem peptides, whereas D,D-carboxypetidases remove the terminal residues from
218 stem peptides. In *E. coli,* none of the class C PBPs are essential for viability. Bacteria lacking
219 all seven of them grow nearly as well as the parental strain and display only modest
220 morphological defects (Denome et al., 1999).

221   Among the three different aPBPs produced by *E. coli*, the best-studied and most
222 important are PBP1a and PBP1b. Despite having the same name as two of the pneumococcal
223 class A PBPs, they belong to different subclasses and are not functionally equivalent or closely
224 related to their pneumococcal counterparts. *E. coli* cells must produce either PBP1a or PBP1b

225 to be viable (Yousif et al., 1985). Like PBP1a and PBP2a from *S. pneumoniae* they are semi-
226 redundant enzymes, i.e. they can substitute for each other with regard to viability under
227 standard laboratory growth conditions but in addition have specific non-overlapping functions.
228 An interesting observation regarding the properties of PBP1a and PBP1b is that their enzymatic
229 activity is influenced by pH. Hence, maximal fitness across a wide pH range (pH 4.8-8.2) seems
230 to require the function of both PBPs (Mueller et al., 2019). PBP1c, the third aPBP encoded in
231 the genome of *E. coli*, cannot substitute for the double loss of PBP1a and PBP1b. Moreover, a
232 PBP1c deletion mutant is viable, and does not show any obvious phenotype (Schiffer & Höltje,
233 1999). The transpeptidase and transglycosylase activities of PBP1a and PBP1b are regulated
234 by their cognate outer-membrane lipoproteins LpoA and LpoB (Typas et al., 2010; Paradis-
235 Bleau et al., 2010; Jean et al., 2014; Egan et al., 2014). PBP1a, which localizes to foci in the
236 lateral cell wall, has been reported to interact with the elongation-specific class B
237 transpeptidase PBP2 (Banzhaf et al., 2012). Hence, this aPBP appears to have an important
238 role in cell elongation. Despite that it is found throughout the cell envelope, PBP1b is
239 considered to be specialized for cell division. It is reported to interact with several divisome
240 proteins, namely PBP3, FtsW, FtsN, FtsQ, FtsL and FstB (Bertsche et al., 2006; Müller et al.,
241 2007; Leclercq et al., 2017; Boes et al., 2019). Thus, all in all, there is considerable evidence
242 that PBP1b is an intrinsic component of the divisome, while PBP1a is an intrinsic component
243 of the elongasome in *E. coli*.

244   Curiously, despite the low amino acid sequence identity of PBP1a and PBP1b (27%),
245 and all the specific interactions reported for these aPBPs with partners in the divisome and
246 elongasome, PBP1a and PBP1b are largely interchangeable in their capacities to support
247 growth. Presumably the change of partners involved would require that both PBPs are able to
248 interface productively with members of both complexes. In other words, both must be able to
249 function as integrated cogwheels in the elongasome as well as divisome machinery. It takes a
250 stretch of the imagination to envisage how this could be possible. In a single-molecule study,
251 Cho et al. (2016) present results that conflict with the model predicting that aPBPs are intrinsic
252 components of the elongasome. Under the experimental conditions used, they found that the
253 RodA/PBP2 complex, but not aPBPs, displays MreB-guided circumferential motion in *E. coli,*
254 demonstrating that the elongasome and aPBPs are spatially distinct. Hence, class A PBPs can
255 be envisioned as dynamic and relatively autonomous entities that are not directly involved in
256 synthesizing the cell wall, but rather have auxiliary functions such as repair and/or fortification
257 of the peptidoglycan layer. A similar view has been put forward by Vigouroux and colleagues

258   (2020). In a recent article, they reported that aPBPs have no role in maintaining the cell shape

259   but were crucial for mechanical cell wall integrity (Vigouroux et al., 2020). Hence, aPBPs

260   evidently function to strengthen the cell wall of *E. coli*. The authors found evidence that this

261   strengthening is due to an adaptive class A-mediated repair mechanism that senses and repairs

262   cell wall defects. In support of this, Lai et al. (2017) found that PBP1b-mediated peptidoglycan

263   synthesis increases following overexpression of the space-maker endopeptidase MepS (Spr),

264   an enzyme that makes room for localized insertion of new material during peptidoglycan matrix

265   expansion (Singh et al., 2012). Their findings strongly indicate that aPBPs and their auxiliary

266   proteins detect and fill gaps in the peptidoglycan network. Taken together, available data

267   suggest that aPBPs serve dual roles. They operate in conjunction with the divisome and

268   elongasome during synthesis of the primary cell wall, but in addition function as autonomous

269   entities that maintain and repair the peptidoglycan sacculus.

270

5 | THE SYNTHETIC LETHALITY PARADOX

272   As discussed above, aPBPs often form synthetic lethal pairs such as PBP1a/PBP2a in

273   *S. pneumoniae* and PBP1a/PBP1b in *E. coli*. Each member of these pairs can be individually

274   deleted, but the bacterium must produce one of them to be viable. Moreover, members of each

275   pair have overlapping as well as non-overlapping functions, demonstrating that they are semi-

276   redundant. It follows from this that it must be their overlapping function that is essential for

277   cell survival. What could this essential function be? The many studies reporting close

278   interactions between aPBPs and proteins associated with the divisome or elongasome, suggest

279   that aPBPs are intrinsic components of these multiprotein complexes (Steele et al., 2011;

280   Scheffers & Errington, 2004; Claessen et al., 2008; Fenton et al., 2016; Bertsche et al., 2006;

281   Leclercq et al., 2017; Boes et al., 2019). On the other hand, strong recent experimental

282   evidence shows that aPBPs are able to function autonomously (Lai et al., 2017; Vigouroux et

283   al., 2020; Straume et al., 2020). How can this apparent paradox be explained? Perhaps aPBPs

284   can operate both autonomously and in a context where their actions are coordinated with the

285   activities of the elongasome and divisome. When they detect and repair damage to the

286   peptidoglycan sacculus localized outside areas of active peptidoglycan synthesis, aPBPs

287   probably function as autonomous entities, possibly together with accessory proteins that help

288   regulate and direct their activity. However, in areas where synthesis of new cell wall

289   peptidoglycan takes place, aPBPs may operate in conjunction with the divisome and

290 elongasome to repair defects made during primary peptidoglycan synthesis. Members of
291 synthetic lethal pairs of aPBPs have low amino acid identity. Hence, it is unlikely that they can
292 substitute for each other in cases where their function depends on close interactions with
293 several divisome or elongasome proteins. However, it is conceivable that they can perform
294 overlapping repair functions when operating as autonomous entities. We therefore propose that
295 the essential function of class A PBPs is to detect and repair gaps and imperfections in the call
296 wall peptidoglycan localized outside areas of active peptidoglycan synthesis.

297

6 | A TWO-LAYERED CELL WALL IN GRAM-POSITIVE BACTERIA?

299 In a newly published study by Pasquina-Lemonche and co-workers (2020), atomic force
300 microscopy was used to study the cell wall architecture of the Gram-positives *B. subtilis* and
301 *S. aureus*. Interestingly, the paper reports evidence that nascent septa in both species consist of
302 two different peptidoglycan layers with distinct architectures that indicate two synthesis
303 regimes (Fig. 1). The inner layer, in the following referred to as the core or primary cell wall,
304 has a highly ordered structure composed of concentric rings. The core is sandwiched between
305 the outer layers, which constitute the bulk of the septum thickness. In contrast to the core, the
306 outer layers (the secondary cell walls) are made up of randomly oriented material (Fig. 1). As
307 the core peptidoglycan in nascent septa acts as a scaffold for the outer layers, the core material
308 must be synthesized first. Hence, the authors suggest that the core peptidoglycan is formed at
309 the leading edge of the cross wall, while the outer layers are subsequently added on top of the
310 core. During cell wall growth and maturation, the internal cytoplasm-facing surface of *S.*
311 *aureus* cells remains a disordered mesh with relatively small pores. In contrast, the post-
312 divisional outer surface, which originally had an ordered ring-like architecture, gradually
313 matures into a porous mesh due to the action of various peptidoglycan hydrolases. Similarly,
314 the outer surface of *B. subtilis* cells reorganizes into a randomly oriented porous network, while
315 the poles retain some of the ring structure originating from the inner core of the split septum.
316 The inner surface of the *B. subtilis* cell-wall cylinder, on the other hand, consists of material
317 that has been deposited in a circumferential orientation (Fig. 1). This is due to the
318 circumferential movement of MreB filaments which guide the movement of the elongasome
319 (Garner et al., 2011; Domínguez-Escobar et al., 2011; Dion et al., 2019; Pasquina-Lemonche
320 et al., 2020). Very recently, a similarly structured two-layered cell wall has also been reported
321 for *Staphylococcus warneri* (Su et al., 2020).

How are the two different layers of nascent septa synthesized? A likely solution to this question involves the function of aPBPs. In the paper by Straume *et al.* (2020) several possible functions for aPBPs were proposed. One of these was that aPBPs make the primary peptidoglycan produced by the PBP2x/FtsW machinery stronger and denser by adding peptidoglycan that is more heavily cross-linked. This hypothesis agrees well with the data presented in the paper by Pasquina-Lemonche *et al.* (2020). Hence, we postulate that the PBP2x/FtsW machinery synthesize the ordered rings deposited at the leading edge of the growing septum, while class A PBPs subsequently adds the randomly oriented peptidoglycan deposited on top of the core material (Fig. 2). The finding that the activity of class A PBPs occurs subsequent to and separate in time from divisome-mediated peptidoglycan synthesis fits nicely with this model (Straume et al., 2020).

Presumably, the question could be settled by examining the *B. subtilis* class A-less mutant by atomic force microscopy along the lines described by Pasquina-Lemonche and co-workers (2020). The fact that *B. subtilis* is able to grow and multiply without aPBPs shows that the FtsW/PBP2b and RodA/PBP2a/PBPH machineries (Table 1) are capable of synthesizing a functional cell wall on their own. SEDS- and aPBP-type glycosyltransferases must have evolved independently at different times during evolution. Thus, early prokaryotes possessing an outer peptidoglycan layer may well have used only SEDS-type glycosyltransferases to build

A two-layered septal cross-wall has not been demonstrated for the pneumococcus yet, but as *B. subtilis*, *S. aureus, S. warneri* and *S. pneumoniae* all belong to class *Bacilli* it is reasonable to assume that their cross-walls are built in a similar manner and have the same architecture (Fig. 1). As stated in Table 1, PBP2 is the only aPBP in *S. aureus*. It is active at the septum as well as in peripheral regions and is thought to cooperate with the PBP1/FtsW machinery to synthesize the septal cross wall. Thus, from what is currently known, it cannot be excluded that PBP2 is responsible for synthesizing the secondary outer layer of the *S. aureus* cell wall. On the contrary, as PBP2 is the only peptidoglycan-synthesizing entity in *S. aureus* apart from the PBP1/FtsW and PBP3/RodA complexes, and only PBP2 and PBP1/FtsW are essential, it is difficult to envision a two-layer-generating synthesis mechanism that does not involve PBP2. Alternatively, the divisome, using different modus operandi, might synthesize two architecturally different layers of peptidoglycan. However, since septum synthesis in *S. aureus* does not take places exclusively at the leading edge but occurs across the whole septal surface (Lund et al., 2018), the latter mechanism seems to be the less likely of the two alternatives.

355 their cell wall. It is conceivable that PBPs with glycosyltransferase domains evolved later in

356 evolution to work in conjunction with the SEDS-type peptidoglycan polymerases as auxiliary

357 proteins. Different theories have been put forward to explain the transition between monoderm

358 (Gram-positive) and diderm (Gram-negative) bacteria. Although this question has not been

359 settled, the latest phylogenomic analyses support a scenario where diderms represent the

360 ancestral type. The monoderm phenotype appears to have arisen independently multiple times

361 due to the loss of key genes involved in the synthesis of the outer membrane (Megrian et al.,

362 2020). Thus, it is likely that the thick peptidoglycan layer surrounding many Gram-positive

363 bacteria has evolved to compensate for the loss of the outer membrane. As outlined above,

364 recent studies on the function of aPBPs in *E. coli* favor a model in which their primary role is

365 to repair and maintain the integrity of the peptidoglycan sacculus. In the Gram-positives, class

366 A PBPs may have acquired a new or additional function to compensate for the stress generated

367 by the loss of their outer membranes, namely to build a thicker cell wall by synthesizing a

368 secondary peptidoglycan layer on top of the primary cell wall made by the FtsW

369 glycosyltransferase and its cognate bPBPs.

370 The recently discovered two-layered architecture of *B. subtilis* and *S. aureus* cell walls

371 raises a pertinent question: how is cell elongation conducted in bacteria with a heterogenous

372 double-layered cell wall? Most likely, elongation of the primary and secondary cell walls

373 would be carried out by separate protein complexes. Different peptidoglycan-cleaving enzymes

374 (space-maker enzymes) are probably required to make room for insertion of new material in

375 each of the two different peptidoglycan layers. Based on the hypothesis that aPBPs synthesize

376 the cytoplasm-facing secondary layer of the septal cross wall, it is conceivable that bifunctional

377 PBPs fill in the gaps made by space-maker enzymes in this part of the cell wall while the RodA

378 complex inserts new material in the primary cell wall. If so, the aPBPs and the RodA complex

379 most likely coordinate their activities to synchronize the elongation process between the two

380 layers.

381 Bacteria produce a large number of peptidoglycan hydrolases which are essential for

382 maintaining the architecture and function of the bacterial cell envelope. They are involved in

383 processes such as cell separation, cell enlargement, recycling of peptidoglycan and assembly

384 of trans-envelope structures too large to pass through the natural pores of the peptidoglycan

385 sacculus (Vollmer et al., 2008b; Scheurwater & Burrows, 2011; Typas et al., 2012). It is not

386 clear how bacteria control the potential suicidal activity of these enzymes, but several different

387 mechanisms are undoubtedly applied. One advantage of evolving a two-layered architecture

may be that it enables the cell to better control the activity of peptidoglycan-degrading enzymes in order to avoid autolysis and cell death. Pasquina-Lemonche *et al*. (2020) reports that in *S. aureus,* the external side of the wall has significantly larger pores than the internal side, and that the large external pores become narrower as they traverse the wall (Fig. 1). Perhaps the activity of the enzymes creating these pores is controlled, at least in part, by the architecture of the double-layered cell wall. The same may be the case for potential suicide enzymes involved in cell separation. PcsB, for example, is the peptidoglycan hydrolase that splits the septal cross wall during pneumococcal daughter cell separation (Bartual et al., 2014). Although PcsB is strictly regulated by the membrane associated FtsEX complex (Sham et al., 2011; Sham et al., 2013, Rued et al., 2019; Alcorlo et al., 2020), its activity may also be controlled by a structurally heterogeneous cell wall. Hence, it is conceivable that PcsB specifically cleaves peptide bridges connecting glycan strands in the circumferentially oriented core layer, while being unable to attack the disordered flanking layers (Fig. 2).

7 | CONCLUSION

The traditional paradigm of peptidoglycan biogenesis states that aPBPs are intrinsic key components of the divisome and elongasome. However, after it was discovered that the SEDS proteins FtsW and RodA have glycosyl transferase activity and work in conjunction with bPBPs, aPBPs were in principle no longer critical components of these multiprotein complexes. Moreover, aPBPs are not essential for survival in some Gram-positive bacteria, strongly indicating that at least in monoderms, bifunctional PBPs are not directly involved in synthesizing the primary cell wall. It is therefore time to rethink and revise the role of aPBPs. Several recent studies in *E. coli* present evidence that aPBPs are important for repair and maintenance of the peptidoglycan matrix. Perhaps *E. coli* produce three different bifunctional PBPs in order to detect and repair different types of damages to the peptidoglycan meshwork. It is likely that aPBPs play a similar repair and maintenance role in Gram-positive bacteria (Fig. 2). In addition, we postulate that aPBPs are responsible for synthesizing the cytoplasm-facing section of the two-layered cell wall described for *B. subtilis*, *S. aureus* and *S. warneri* (see Fig. 2) (Pasquina-Lemonche et al., 2020; Su et al., 2020). So far, a two-layered cell wall has only been demonstrated for these species, but there is no reason why it should not be widespread among Gram-positive bacteria.

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Table 1. Overview and comparison of important cell wall building proteins from the model
organisms *S. pneumoniae*, *S. aureus*, *B. subtilis* and *E. coli*. Only proteins that are mentioned
in this review have been included.

Proteins				Function of proteins
S. p.[a]	*S. a.*[b]	*B. s.*[c]	*E. c.*[d]	
PBP1a PBP2a	PBP2	PBP1	PBP1a PBP1b	Most important class A PBP(s) in the species listed. They are bifunctional and have transpeptidase as well as transglycosylase activity.
PBP1b	–	PBP4	PBP1c	Class A PBPs that can be inactivated without appreciable effect on growth and morphology.
–	–	PBP2c PBP2d	–	Class A PBPs involved in the synthesis of spore peptidoglycan.
PBP2b	PBP3	PBP2a PBPH	PBP2	Class B PBPs that work together with RodA in the elongasome to synthesize peptidoglycan along the lateral cell body. Class B PBPs are monofunctional and have only transpeptidase activity.
PBP2x	PBP1	PBP2b	PBP3	Class B PBPs that work together with FtsW in the divisome to synthesize the septal cross-wall.
–	–	PBP3	–	Non-essential class B PBP of unknown function. Becomes essential when PBP2b of *B. subtilis* is catalytically inactive.
–	–	SpoVD PBP4b	–	Class B PBPs involved in the synthesis of spore peptidoglycan.
[1]PBP3	[1,3]PBP4	[2]PBP4* [1]PBP4a [1]PBP5 [1]PBP5* [1]DacF [2]PBPX	[1,2]PBP4 [1]PBP4b [1]PBP5 [1]PBP6 [1]PBP6b [2]PBP7 [1,2]AmpH	Class C, low-molecular-weight PBPs, that have carboxypeptidase[1], endopeptidase[2] or transpeptidase[3] activity. Some have more than one type of enzymatic activity. Carboxypeptidases regulate the extent of cross linking in peptidoglycan by removal of terminal residues from pentapeptides side chains. Endopeptidases cleave within the peptide chains that form the cross-links between glycan strands in peptidoglycan.
FtsW	FtsW	FtsW	FtsW	SEDS family peptidoglycan polymerase (GTase). Part of the divisome where it works in conjunction with its accompanying class B PBP.
RodA	RodA	RodA	RodA	SEDS family peptidoglycan polymerase (GTase). Part of the elongasome where it works in conjunction with its accompanying class B PBP.
FtsB	FtsB	FtsB	FtsB	FtsB, FtsL and FtsQ form a subcomplex that constitutes the core of the bacterial divisome. The FtsBLQ subcomplex interacts with several divisomal proteins and has a regulatory role in the initiation of septal peptidoglycan synthesis. It inhibits the FtsW/bPBP machinery until the time is right to divide.
FtsQ	FtsQ	FtsQ	FtsQ	
FtsL	FtsL	FtsL	FtsL	
FtsN	FtsN	FtsN	FtsN	FtsN relieves FtsBLQ-mediated inhibition of the FtsW/bPBP machinery.
MurJ	MurJ	MurJ	MurJ	Transporter that flips lipid II across the cytoplasmic membrane.
CozE	CozE	CozE	CozE	Broadly distributed polytopic membrane protein that together with PBP1a and MreCD coordinates cell elongation in *S. pneumoniae*. In *S. aureus* it has been reported to control cell division.
CozEb	CozEb	CozEb	CozEb	Homolog of CozE that contributes to cell size homeostasis in *S. pneumoniae*.
GpsB	GpsB	GpsB	–	In *S. pneumoniae* and *B. subtilis* GpsB acts as an adaptor that coordinates peptidoglycan synthesis with other processes in a cell cycle-dependent manner. In *S. aureus* GpsB stabilizes the Z-ring at the onset of cell division and stimulates cytokinesis through direct interaction with FtsZ.
–	–	MreB	MreB	The actin homolog MreB directs lateral cell wall synthesis in rod shaped bacteria.
MreC	MreC	MreC	MreC	Associated with the elongasome. May act as a scaffold for other components of the elongasome machinery.
MreD	MreD	MreD	MreD	Polytoptic membrane protein that is part of the elongasome. Regulatory and/or scaffolding function.
RodZ	RodZ	RodZ	RodZ	Required for cell-elongation in rod-shaped and ovoid bacteria. Forms a supramolecular complex with elongasome proteins such as MreB (if present), MreC, MreD and peptidoglycan synthases. The role of RodZ in *S. aureus* has not been determined.

MacP	–	–	–	Membrane anchored substrate of StkP, and an activator of PBP2a.
PcsB	–	CwlO	AmiA AmiB	Peptidoglycan hydrolases that split the septal cross-wall during cell division and are regulated by FtsEX (PcsB) or FtsEX/EnvC (AmiA and AmiB). CwlO is regulated by FtsEX and are involved in regulating growth and cell elongation.
FtsE	[4]FtsE	FtsE	FtsE	ATPase required for the transmission of a conformational signal from the cytosol through the membrane via FtsX. In *S. pneumoniae* and *E. coli* FtsEX regulates the activity of cell wall hydrolases that cleave the septum to release daughter cells after cell division. Instead of controlling cell division, *B. subtilis* FtsEX controls the peptidoglycan hydrolase CwlO, which plays a central role in cell wall elongation during growth. [4]Little research has been conducted to investigate the presence and potential role of FtsEX-like proteins in *S. aureus*. It is therefore uncertain whether *S. aureus* contains a FtsEX-system corresponding to those regulating cell division in *S. pneumoniae* and *E. coli* and cell wall elongation in *B. subtilis*.
FtsX	[4]FtsX	FtsX	FtsX	The transmembrane protein FtsX mechanically transduce a conformational signal from the cytoplasmic FtsE that provokes the activation of peptidoglycan hydrolases.
–	–	–	MepS (Spr)	Endopeptidase that cleaves peptidoglycan during cell wall expansion to allow insertion of new glycan strands.
MltG	–	MltG	MltG	Lytic transglycosylase proposed to be responsible for glycan strand termination during peptidoglycan synthesis.
CbpD	–	–	–	Competence induced peptidoglycan hydrolase.
StkP	PknB	PrkC	–	Eukaryotic-like serine/threonine kinase that has been reported to sense lipid II and peptidoglycan fragments. StkP and PknB regulate cell division and peptidoglycan synthesis in *S. pneumoniae* and *S. aureus,* respectively. In *B. subtilis* PrkC does not affect cell division, morphology or cell growth, but alters stationary phase physiology and induces spore germination. No ortholog with a PASTA domain is present in *E. coli*.
–	–	–	LpoA	Outer-membrane-anchored lipoprotein that regulates the function of PBP1a.
–	–	–	LpoB	Outer-membrane-anchored lipoprotein that regulates the function of PBP1b.
FtsZ	FtsZ	FtsZ	FtsZ	Structural homologue of tubulin that forms a cytokinetic ring at mid-cell and recruits the division machinery to orchestrate cell division.

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[a]*Streptococcus pneumoniae*, [b]*Staphylococcus aureus*, [c]*Bacillus subtilis*, [d]*Escherichia coli*
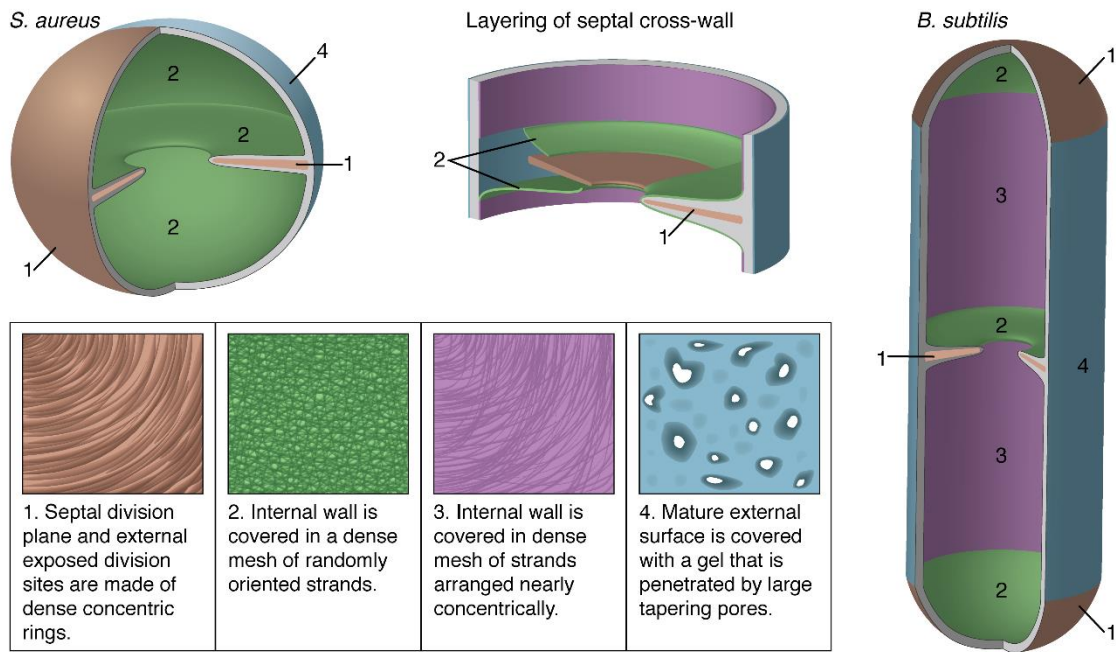
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Fig. 1. Architecture of the cell walls of *S. aureus* and *B. subtilis* as proposed by Pasquina-Lemonche and co-workers (2020). The figure is adapted from extended data Fig. 10 in their article.
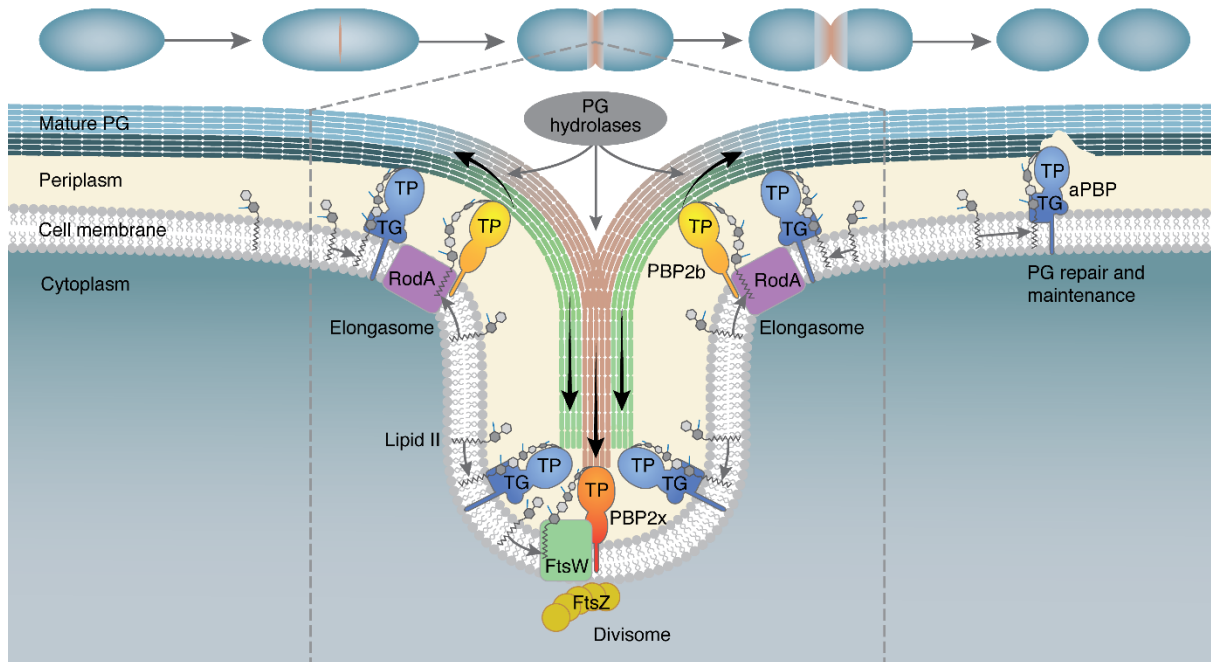
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Fig. 2. Model that depicts possible roles of aPBPs in the construction and maintenance of a two-layered cell wall in *S. pneumoniae*. SEDS proteins (FtsW and RodA) in conjunction with bPBPs (PBP2x and PBP2b) represent the primary peptidoglycan-synthesizing machineries that build the septum (divisome) and insert new material into the lateral cell wall during cell elongation (elongasome). It has recently been reported that the septal cross wall of Gram-positive bacteria appears to consist of two peptidoglycan layers with different architectures (Pasquina-Lemonche et al., 2020). Based on this and our own data (Straume et al., 2020), we postulate that the FtsW/Pbp2x machinery of the divisome synthesize the highly ordered core layer of the cross-wall (brown) which is matured (light blue) by the action of peptidoglycan (PG) hydrolases, while aPBPs working in conjunction with the divisome synthesize the disordered peptidoglycan layer facing the cytoplasm (green). Furthermore, as illustrated by the aPBP operating outside the midcell region, new evidence suggests that aPBPs can function as autonomous entities that are involved in repair and maintenance of the peptidoglycan sacculus.